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EVOLUTION AND FUNCTION OF STRUCTURALLY DIVERSE SUBUNITS IN THE RESPIRATORY PROTEIN HEMOCYANIN FROM ARTHROPODS

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ABSTRACT

Native aggregation level and subunit composition of the hemocyanins from 86 adult chelicerates and crustaceans, and from the larval stages of 2 crabs, were analyzed by means of electron microscopy, polyacrylamide electrophoresis, immuno blotting, and crossed immunoelectrophoresis, supported by a variety of preparative separation techniques. The up to eight immunologically discernible subunit types were interspecifically correlated, classified, and evolution lines derived. Phylogenetic consequences are discussed, and are particularly aggravating in spiders.

A single subunit suffices for the formation of hexamers (1×6). In the architecture of higher-ordered hemocyanins, the various subunits act as building-blocks of distinct specification. This was studied in 2×6 molecules from a hunting spider and several crustaceans, and in 4×6 hemocyanin from a tarantula. The various subunits are present in constant proportions. The total set is required to reorganize the original aggregate from subunit mixtures. Stable oligomeric segments of native hemocyanin particles revealed the gross distribution of the diverse subunits. Immuno electron microscopy of the native hemocyanins decorated with monospecific Fab fragments showed the exact topographic position of each subunit type, and detailed models of the quaternary structure could be derived.

The oxygen binding function of 4×6 hemocyanin from the tarantula *Eurypelma californicum* is excessively modulated by subunit interaction phenomena. We measured native, reassembled, and mercury-blocked 4×6-mers, oligomeric segments, single subunits, and reassembled 4×6-mers with one subunit type chemically modified. The spatial range of allosteric interaction, and specific contributions of the diverse subunits are outlined.

INTRODUCTION

Hemocyanin, a blue copper-protein, functions as an oxygen carrier in the blood of arachnids, horseshoe crabs, crustaceans, and centipedes. Another hemocyanin occurs in inkfishes, chitons, many snails, and some primitive bivalves; however, despite certain similarities, molluscan hemocyanin differs decisively from arthropod hemocyanin in its main structural features, and therefore will not be discussed here. Most certainly, both pigments have evolved independently from tyrosinase, an ancestral precursor. Arthropod hemocyanins are multi-subunit proteins with a molecular mass of about 75,000 per polypeptide chain. The polypeptides—or subunits—are arranged as cubic hexamers (1×6), or multiples of hexamers (2×6, 4×6, 6×6, 8×6); the native aggregation level is species-specific. (Ghiretti, 1968; Van Holde and van Bruggen, 1971; Van Holde and Miller, 1982; van Bruggen *et al.*, 1982; Ellerton *et al.*, 1983;

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Huber and Lerch, 1986; Mangum *et al.,* 1985; Morse *et al.,* 1986). In molecular mass, the 8 × 6-mer (48 subunits) exceeds human hemoglobin 50-fold, and measures about 25 nanometers across, corresponding to the size of a ribosome. The three-dimensional structure of the basic hexamer recently was elucidated in detail (Gaykema *et al.,* 1984). All hemocyanin aggregates are clearly visible in the electron microscope (Fig. 3); the arrangement of hexamers in the higher-ordered molecules, as shown in Figure 1, was partially derived by computer image analysis (Van Heel and Frank, 1981; Bijlholt *et al.,* 1982). Hemocyanin is not incorporated in cells, due to physical problems (Mangum, 1985), but floats freely in the blood in concentrations up to 120 mg/ml (scorpions), meaning that it fills up to $^1/_{10}$ of the hemolymph space; the average distance from one molecule to the next equals its own diameter. Arthropod hemocyanin particles dissociate at alkaline pH into their subunits, which are capable still of reversibly binding oxygen. By dialysis of the obtained subunit mixtures against neutral pH, a self-assembly at least of hexamers, and frequently also of higher-ordered molecules occurs.

Each subunit is a delicate structure of around 10,000 atoms: 620 amino acids, which form three domains containing the binuclear copper site, 20 alpha helices, and a large seven-stranded beta barrel (Gaykema *et al.,* 1984; Linzen *et al.,* 1985). At first glance this appears to be a considerable luxury—placing two metal atoms in the position to bind one molecule of oxygen in the respiratory organs, and to free it again in an oxygen-consuming tissue. The red hemoglobin and a derivative, the green chlorocruorin, achieve this with only 140 amino acids per polypeptide chain and thus attain, with the same effort of protein material, a four-fold oxygen binding capacity. The third known group of respiratory proteins, the pink hemerythrins, with a five-fold oxygen transport capacity, does an even better job in this respect. Therefore, one might suggest that hemocyanin, which in most groups was inefficient to compete with the more economical pigments, is a dead-end product of evolution. However, looking at their distribution among the Invertebrata, we observe that hemocyanin occurs excessively in highly complex animals whereas the more primitive phyla dispose of either hemoglobin, chlorocruorin, or hemerythrin (Prosser, 1973; Bonaventura and Bonaventura, 1980). This is surprising because extracellular hemoglobins as well as red blood cell hemoglobins occur sporadically in molluscs as well as in arthropods (foremost in lower taxa), and thus have been available phylogenetically. What possible advantage made hemocyanin successful over hemoglobin in presumably 100,000 highly advanced animal species, despite its considerably lower oxygen binding capacity? This can be speculated at least for arthropods. As with hemoglobin, the reversible oxygen binding of the blue protein is enhanced, modulated, and adapted by so-called "allosteric" phenomena, based on complex interactions between subunits. Arthropod hemocyanins display this functional plasticity to an extent unknown for hemoglobin or any other allosteric protein (Loewe, 1978; Mangum, 1985). Moreover, no hemoglobin found in a possible candidate for the ancestral arthropod or mollusc exhibits an appreciable allosteric behavior. Among the Invertebrata, the heme proteins with more than token cooperativity are, with a single exception (the bivalve *Scapharca inaequivalvis:* Ascoli *et al.,* 1986), the extracellular ones, which invariably occur in the more specialized groups in a particular taxon. So the ancestors of the hemocyanin-containing molluscs and arthropods were really choosing between a functionally inflexible intracellular hemoglobin (high to moderate oxygen affinity, essentially uncooperative and pH-, inorganic ion-, and organic cofactor-insensitive) and a highly plastic (with respect to oxygen affinity and pH dependence), moderately to highly cooperative, inorganic ion and organic cofactor modulable extracellular hemocyanin. To make the decision easier, it is harder to push a red blood cell-containing hemolymph around in an arthropodan or molluscan circulatory system that lacks capillaries (Mangum, 1985).

Vertebrates evolved a special intracellular hemoglobin which is modulable to some degree, and which compensates for its limited functional plasticity with strong ventilation and circulatory systems which continuously create high gas diffusion rates. Moreover, their interior regulation is efficient enough to embed the hemoglobin in permanent homeostasis, which protects it against drastic changes of the *milieu*. As a result, their blood gained the extremely high oxygen carrying capacity of intracellular hemoglobin. The higher arthropods display sensory and locomotory activities comparable to those of many vertebrates. As in vertebrates, these activities require a continuous, abundant oxygen supply of the involved tissues, despite the more limited vegetative control mechanism in these animals and despite their gills and lungs being lined with chitin that hinders gas diffusion. Their respiratory protein thus plays a much more decisive role as a molecular interface between body tissues and environment. Extremely flexible in function, arthropod hemocyanin compensates for environmental and physiological changes during the life-cycle of the organism (Mangum, 1980; Mangum, 1983), and it also has demonstrated flexibility in the evolution of countless crabs, crayfishes, shrimps, isopods, scorpions and spiders, each adapted to a special aquatic or terrestrial situation.

The "clue" to arthropod hemocyanins is their multigenicity: they are composed of several structurally and functionally different types of subunits. This has been studied intensively worldwide during the last decade (for review: Linzen, 1983). The phylogeny of those subunits, and of the various oligomers, is now well understood. In addition we learned that the native aggregation level, and also most probably the oxygen binding characteristics, are ultimately dependent on the special subunit composition.

Materials and Methods

Animal sources, and the specificity of the applied antisera (raised in rabbits) are described elsewhere (Markl *et al.*, 1986a). Other methods are described in detail in the cited literature. However, for a better understanding of the following discussion, a brief description of the major immunochemical approaches may be useful. Immunochemists use very powerful and sensitive techniques (*e.g.*, radioimmunoassay, enzyme-linked immunoassay) which enables the calculation of immunological differences between proteins in 1% steps by successively quantitating the immune reaction between each of the proteins and a suited antiserum. Unfortunately, in our case those methods were inadequate because the hemocyanin subunit samples, especially from crustaceans, were uncontrollably contaminated by self-reassembled aggregates (if the dissociation conditions were too gentle), or by partially denatured subunits (if we chose more drastic conditions). Those contaminants display special immune reactions: compared to subunits, aggregates react at least ten-fold stronger, and denaturation, (*e.g.*, with urea) spirited away intraspecific as well as interspecific immunological differences between hemocyanin subunits (Lamy *et al.*, 1981a; Stöcker, 1984; Kempter *et al.*, 1985). Therefore our data would have been seriously falsified had we used a "blind" quantitative method.

Crossed immunoelectrophoresis

We instead applied the crossed immunoelectrophoresis which, though only being semi-quantitative and less sensitive (soluble immune complexes escape the detection), allows a selective comparison of the desired components in a particular sample. This two-dimensional technique (Weeke, 1973) in the first dimension separates proteins in an agarose gel according to charge differences. In the second dimension the proteins

migrate, again electrophoretically, into an antiserum-containing agarose gel. Each protein which reacts with the antiserum forms a curved precipitation line; intersecting lines allow the structural comparison of the respective proteins. In all the patterns discussed here, the anode was on the left in the first dimension. Variations of this technique (tandem-crossed and crossed-line immunoelectrophoresis) enable an intersection of proteins from two different samples.

Immuno blotting

Especially for comparisons of phylogenetically distant subunits, the more sensitive immuno blotting ("Western blotting") technique was used (Towbin *et al.*, 1979; Burnette, 1981). The native subunit patterns obtained by polyacrylamide gel electrophoresis were transferred by diffusion (the currently used electro transfer caused denaturation!) onto an immobilizing nitrocellulose sheet, and then treated with subunit-specific rabbit antibodies. A second goat antiserum against rabbit antibodies was applied subsequently. The second antibodies carry pick-a-back horseradish peroxidase molecules. This enzyme catalyzes a reaction with added diamino benzidine, resulting in a brownish color. Specifically, only those subunit bands which had reacted with the first antibody are stained, whereas other subunits remain invisible. The weak peroxidase activity of hemocyanin (Ghiretti, 1968) was low. This technique is advantageous as soluble immune complexes also are recorded. However, immuno blotting could not serve us exclusively because it was unable to distinguish slight differences between closely related subunits; for this, crossed immunoelectrophoresis was the better tool.

Hemocyanins of the Arachnida and of Allied Groups

Extent of subunit diversity in tarantula hemocyanin

Ten years ago, when we decided to study the blue blood protein of the North American tarantula, *Eurypelma californicum*, a 4×6-mer, few and very contradictory data were available on the number of subunit types present in any one hemocyanin. Polyacrylamide gradient slabgel electrophoresis is an extremely powerful technique used to separate a mixture of very similar proteins. In a buffer system which separates proteins primarily according to charge differences (molecular mass differences played an additional, but minor role), the subunit mixture of tarantula hemocyanin yielded 6 distinct bands: 5 monomers (= single polypeptides) and a dimer. Using a combination of gel chromatography, ion exchange chromatography, and preparative polyacrylamide electrophoresis (today we use immuno affinity chromatography in one single step), each of the six components could be preparatively isolated. In a detergent-containing polyacrylamide gel, which separates polypeptides according to molecular mass differences, we detected that the dimeric subunit is composed of two different monomers (Schneider *et al.*, 1977; Markl *et al.*, 1979a). Thus, the final result was a complex pattern of seven polypeptide chains, which we later have designated as *a* through *g* (Fig. 1). Also a third method visualized the marked diversity of those subunits, namely the crossed immunoelectrophoresis (Fig. 1). No immunological cross-reactivities between any two subunits could be detected (Lamy *et al.*, 1979a). This indicated that the surface structures must be significantly different, which implied major differences in function.

Distribution of the 4×6-mer and its derivatives

We found no hemocyanin in the sun spider ("wind scorpion") *Galeodes* sp., and in the watermite *Hydrachna geographica* (Markl *et al.*, 1986a). Both animals possess

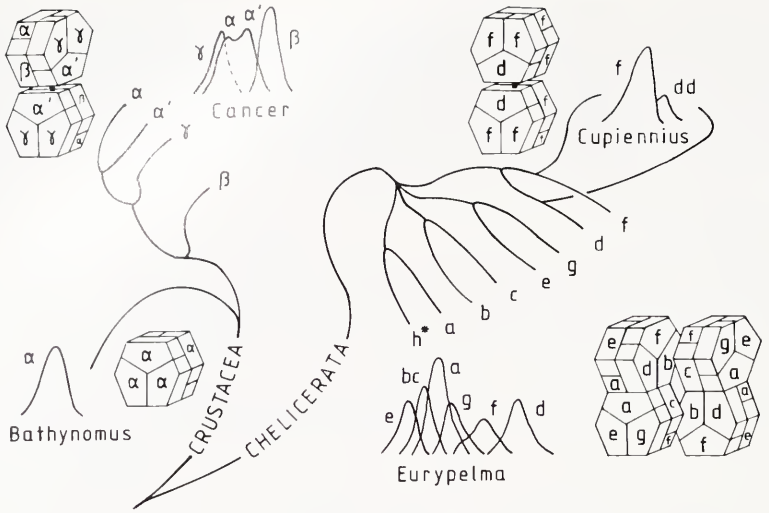

FIGURE 1. Topographical models of the quaternary structure of four arthropodan hemocyanins. The phylogenetic tree shows the relationships between the various subunit types (Kempter *et al.*, 1985; Markl *et al.*, 1986a). The subunits are visualized in their respective patterns of crossed immunoelectrophoresis. Subunit topographies are based on electron microscope analyses of the overall morphology (Van Holde and van Bruggen, 1971; Markl, 1980; Bijlholt *et al.*, 1982; van Bruggen, 1983), and were derived from the combined results of four different approaches: (i) determination of subunit compositions and stoichiometries, (ii) analysis of oligomeric dissociation fragments in comparison with the whole molecule, (iii) reassembly experiments with various subunit combinations, (iv) direct observation in the electron microscope after having decorated the native particles with subunit-specific antibodies (respectively antibody fragments). Beyond the 1×6 level, for the formation of the original aggregate the presence of all subunit types is ultimately required. This reflects perfectly the distinct structural roles played by these subunits.

*: Subunit *h* is restricted to scorpion hemocyanin (see Fig. 2).

1×6 hemocyanin of the deep sea isopod *Bathynomus giganteus* (a gift of M. Brenowitz) is exclusively composed of alpha subunits (Van Holde and Brenowitz, 1981, and unpub. data).

2×6 hemocyanin of the spider *Cupiennius salei* is composed of a disulfide-bridged dimer *d-d* and 10 monomers *f* (Markl, 1980). 2×6 hemocyanin of the crab *Cancer pagurus* consists of a non-covalent dimer alpha'-alpha', a central 4-beta cluster, and, more peripheral, 2 alpha and 4 gamma subunits. A certain flexibility in substituting gamma subunits for alpha appears to exist. 2×6 hemocyanin from the freshwater crayfish *Astacus leptodactylus* is constructed correspondingly, but contains a disulfide-bridged dimer. The crab *Callinectes sapidus* and the lobster *Homarus americanus* fit into the scheme, although in those cases no dimer could be identified (Markl *et al.*, 1983; Stöcker *et al.*, 1986).

4×6 hemocyanin of the tarantula *Eurypelma californicum* is constructed of a central tetrameric *bc-bc* ring, symmetrically surrounded by 20 monomers: one *a, d, e, f,* and *g* in each basic hexamer. Most probably, peripheral bridges between the two 2×6-meric halves are formed via *f-f*. According to Markl *et al.* (1981d). Comparable results stem from scorpion and xiphosuran hemocyanins (Lamy *et al.*, 1981b, 1983b).

a well-developed trachea system and therefore probably have no need of a respiratory pigment. However, as indicated in Figure 2, 4×6-mers composed of multiple subunits are present in whip scorpions and whip spiders (Markl *et al.*, 1978, 1979b). A breakthrough like that made with *Eurypelma* hemocyanin was achieved in the analysis of the 4×6 hemocyanin of another arachnid, the scorpion *Androctonus australis,* and the 8×6 hemocyanin of a xiphosur, the horseshoe crab *Limulus polyphemus* (Hoylaerts *et al.*, 1979; Lamy *et al.*, 1979b, c; Markl *et al.*, 1979b; Brenowitz *et al.*, 1981). *Androctonus* hemocyanin contains an eighth subunit type, designated by us as *h,* and *Limulus* hemocyanin shows two variations of subunit *g,* but for the rest both molecules are composed like *Eurypelma* hemocyanin (Fig. 2). This correspondence was recently confirmed by comparative immunochemistry: each subunit of the tarantula has a

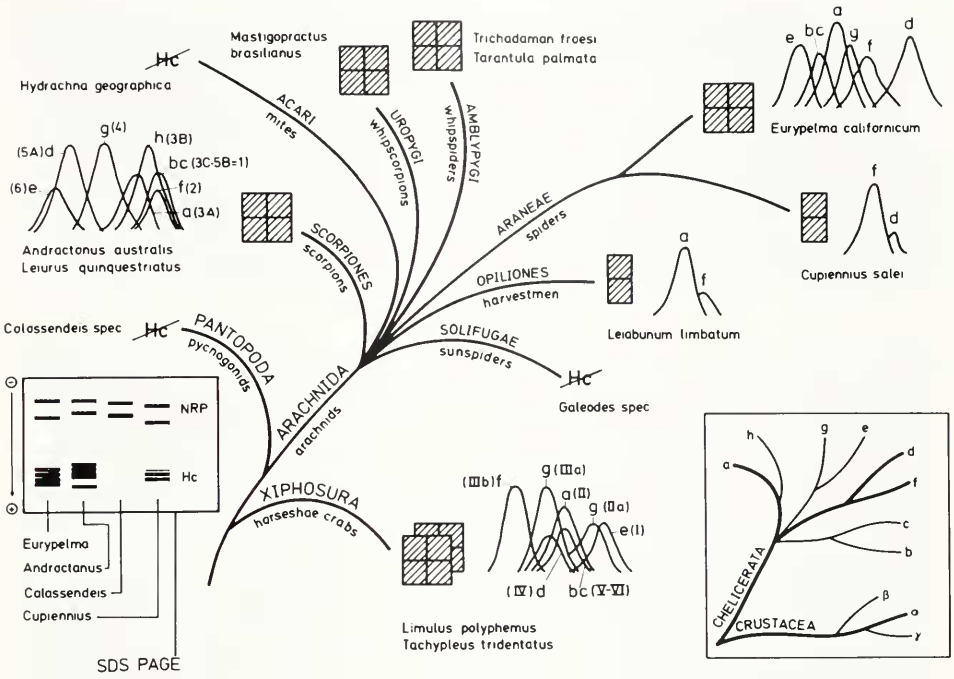

FIGURE 2. Distribution of hemocyanin among the subphylum Chelicerata. The patterns of crossed immunoelectrophoresis show the respective subunit composition; hatched squares symbolize the native aggregation states (8×6, 4×6, 2×6; small squares correspond to hexamers *). Immunologically homologous subunits are identically labeled using the designations *a–h* established for spider hemocyanins. Scorpion and xiphosuran subunits are additionally labeled according to their original designations (Lamy *et al.*, 1979a, b, c; Markl, 1980). The results from *Tachypleus* and *Leiurus* (Lamy *et al.*, 1979c; Markl *et al.*, 1984) are not discussed in the text, but fit well into the scheme. Whip spider and whip scorpion hemocyanin subunits were studied only by electrophoresis (Markl *et al.*, 1979b). Opilionids possess 2×6 hemocyanin (Kempter *et al.*, 1985), whereas no hemocyanin was found in *Galeodes, Hydrachna,* and *Colossendeis* (Markl *et al.*, 1986a). The figure is taken from Markl *et al.* (1986a).

Left insert: polyacrylamide gel electrophoresis (PAGE) of hemolymph proteins in the presence of a detergent (sodium dodecyl sulphate = SDS), showing that the sea spider *Colossendeis* possesses no hemocyanin (Hc), but it does possess the typical arachnidan non-respiratory protein (NRP). This was confirmed by immuno blotting. Comparable unpublished results were recently obtained with the pycnogonid *Nymphon* sp.

Right insert: phylogenetic relationships between the eight cheliceratan subunits according to the combined information of Lamy *et al.*, 1983a, Markl *et al.*, 1984, and Kempter *et al.*, 1985. Broader lines indicate that with anti-crustacean alpha antiserum, subunits *a, d,* and *f* are recognized best.

* For better comparison with the current literature: the sedimentation coefficients of the four aggregates are 60S ($8 \times 6 = 48$-mer), 35S ($4 \times 6 = 24$-mer), 24S ($2 \times 6 = 12$-mer), and 16S ($1 \times 6 = 6$-mer).

homologous subunit in scorpion and horseshoe crab hemocyanin (Lamy *et al.*, 1983a; Markl *et al.*, 1984; Kempter *et al.*, 1985). Moreover, a phylogenetic tree of the subunits could be derived: the pairs *d/f, e/g, b/c,* and *a/h,* respectively, are phylogenetically closely connected (Fig. 1, and right insert in Fig. 2).

Although at that point the various subunits still could be neutral features that have been inherited because they weren't selected against, the heterogeneity appeared as a basic structural design worth conserving in evolution at least since that time when the progenitors of arachnids and xiphosurs diverged from each other. Fossils show that this happened during the Siluran era, more than 400 million years ago (Tiegs and Manton, 1958; Bergström *et al.*, 1980). All the more surprisingly, 4×6 hemocyanin

was discarded twice in rather advanced arachnidan groups, and replaced by relatively simple 1 × 6 and 2 × 6 hemocyanins (Fig. 2). This occurred in the harvestmen (daddy-long-legs; Kempter *et al.*, 1985) and, independently, in certain spiders (Wibo, 1966; Markl *et al.*, 1976, 1983, 1986a). This phenomenon has been studied intensively, especially in spiders. The blood of the large Central American hunting spider *Cupiennius salei* contains both 1 × 6 and 2 × 6 hemocyanin. The 1 × 6-mers showed electrophoretically 5 different subunit bands. However, to our suprise, these components were completely identical immunologically (Markl and Kempter, 1981a). All five could be correlated immunologically with subunit *f* of *Eurypelma* (Markl *et al.*, 1984; Kempter *et al.*, 1985). 2 × 6-mers, present as a main component of *Cupiennius* blood, are also composed of *f*-homologons, but also contain a dimeric subunit (Fig. 1). This dimer is immunologically related to, but somewhat distinct from the monomers (Markl, 1980) and, as revealed recently by immuno blotting, predominantly related to *Eurypelma* subunit *d* (Markl *et al.*, 1986a). Thus, the 2 × 6 hemocyanin of *Cupiennius* can be derived phylogenetically from a 4 × 6-mer progenitor.

The architecture of Cupiennius *hemocyanin*

Parallel to the comparative studies described so far, we attempted from the beginning to analyze hemocyanin subunits with respect to their reassembly behavior, native stoichiometry, and topologic position in the respective oligo-hexamer. Our goal was to reveal principles of the blue protein's architecture. We used a variety of analytical methods, but especially polyacrylamide gel electrophoresis, immunochemistry, and electron microscopy. Each of the monomeric *f* isozyms of *Cupiennius salei* hemocyanin formed regular homo-hexamers in reassembly experiments; to reorganize the dodecamer, the disulfide-bridged dimer *dd* also was required (Markl, 1980; Markl and Kempter, 1981a). Stoichiometrically, each 2 × 6 particle contained one copy of *dd* and ten copies of *f*. Electron microscopy indicated that a one-point contact between the two hexameric halves could be assumed (Fig. 1). Reducing agents, which cleaved the isolated dimer into single *d* monomers, also cut the 2 × 6-mer down to hexamers. Together, these techniques strongly indicated, but only indirectly, that the dimer was an inter-hexamer bridge—how could this be tested more rigorously? Immuno labeling experiments, as described below, were unsuccessful because of the marked immunological similarity between *Cupiennius dd* and *f*. The solution came unexpectedly: when we monitored the dissociation of a 2 × 6 fraction electrophoretically, we observed that the process passed over a stable oligomeric fragment of unusual size. Molecular mass determinations characterized it as heptamer. In a carefully directed electron microscopic survey we indeed detected a strange, yet undescribed molecule: a regular hexamer with a protruding particle (Fig. 3). We isolated this heptamer and, by reducing agents, cut off the protrusion—obviously a fragment of the molecular bridge. The products of this cleavage were hexamers and single subunits *d*. Thus indeed, the protrusion was identified as the half of the dimer. The resulting model of quaternary structure (Fig. 1) was, among all hemocyanin models, the first which showed a directly localized subunit type in its topologic position (Markl, 1980).

The 4 × 6-mer—a symmetrical mosaic

Computer analysis of electron microscope images revealed the general morphology of *Eurypelma californicum* hemocyanin particles (Bijlholt *et al.*, 1982): the two basic hexamers within a half-structure are rotated against each other in a right angle and come in close contact; the 2 × 6-meric halves keep more distance and are slightly tilted with respect to each other (Fig. 1).

Since two subunit types form a 2 × 6-mer, the necessity of five more different components to make the protein twice as large was difficult to see, and we asked

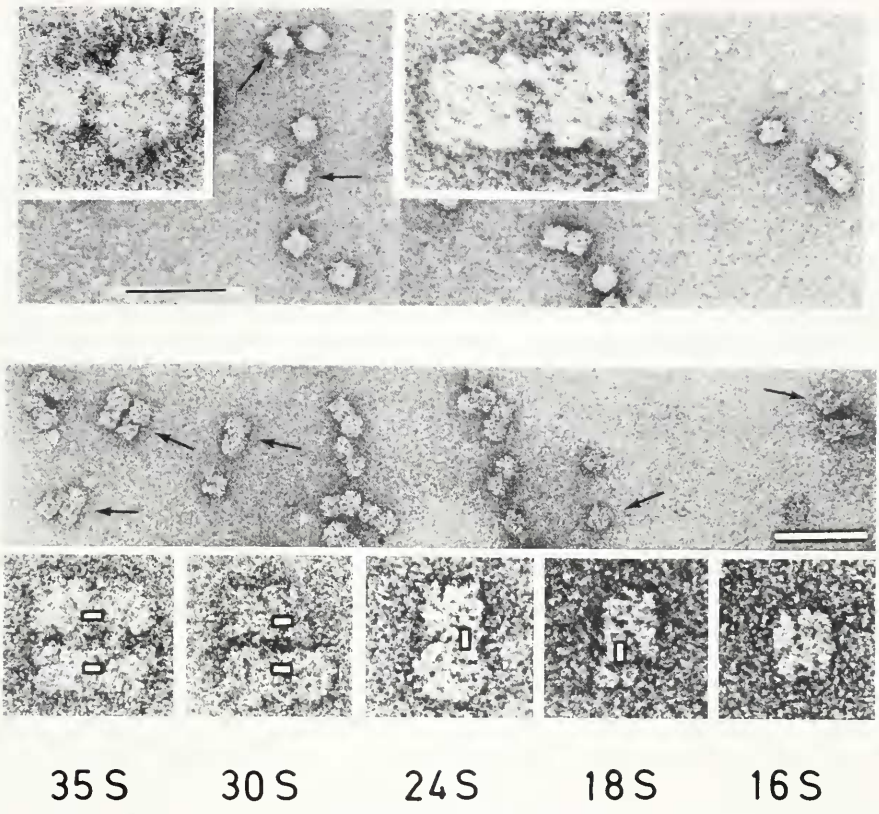

FIGURE 3. *Above:* electron micrographs of hemocyanin molecules from the spider *Cupiennius salei*, negatively stained with 1% unbuffered uranyl acetate. Besides 1 × 6 and 2 × 6 aggregates, heptameric intermediate structures are visible (arrows). The bar represents 50 nm. The left magnified image shows a heptamer with its protruding particle, which could be identified as half-dimer *d*. The right enlargement shows a 2 × 6 molecule in the same orientation. From Markl (1980).

Below: electron micrographs of negatively stained hemocyanin particles from the tarantula *Eurypelma californicum*. The molecules were obtained after 3 hours dialysis of native 4 × 6-mers against pH 9.6, which caused a partial dissociation into oligomeric fragments (upper panel, the bar represents 50 nm). The magnified images (lower panel) have the position and orientation of the heterodimeric subunit *bc* indicated, and are from left to right: 4 × 6-mer (= 24-mer, sediments in the analytical ultracentrifuge with 35S); 3 × 6-mer with one additional subunit protruding in the gap (= 19-mer, 30S); 2 × 6-mer (= dodecamer, 24S); 1 × 6-mer with an additional subunit protruding (= heptamer; 18S); 1 × 6-mer (= hexamer, 16S). The figure is taken from Markl *et al.*, 1981c.

ourselves whether all of these components are really incorporated in the same hemocyanin particle. Using various analytical methods, we tried to detect a heterogeneity of the 4 × 6 molecules of *Eurypelma;* finally we were convinced that indeed only a single type of 4 × 6-mer exists (Markl *et al.*, 1980). The 7 subunits, designated *a* through *g*, are present in constant proportions: $4a + 2b + 2c + 4d + 4e + 4f + 4g = 24$ (Markl *et al.*, 1980, 1981a). The self-assembly process of the 4 × 6-mer is characterized by a striking specificity: reassembled molecules equal native molecules in the stoichiometry of their subunit composition (Decker *et al.*, 1980). None of the subunits was able to self-assemble to homo-hexamers, in contrast to certain horseshoe crab and scorpion hemocyanin components (Lamy *et al.*, 1977; Bijlholt *et al.*, 1979; Brenowitz *et al.*, 1983). A series of reassembly experiments with all possible subunit combinations revealed that indeed each subunit type is required to build up the oligo-

meric architecture. A substitution by another subunit is not successful: the reassembly process will stop at a certain level, which is typical for each subunit combination (Markl *et al.*, 1981b, 1982). Most of the intermediate structures obtained morphologically corresponded to one of the dissociation fragments described below (Fig. 3). Stable 4 × 6 particles, with a "normal" appearance in the electron microscope are only reorganized in the presence of all seven components. After this, our goal was to localize each subunit within the native 4 × 6 particle. Again, an essential part was the investigation of stable oligomeric dissociation fragments. We attempted a carefully directed search for heptamers, which we indeed found (Fig. 3), together with "conventional" dodecamers and hexamers (Markl *et al.*, 1981c). Additionally, a new fragment appeared: a 19-mer, composed of 3 hexamers and a small protrusion in the gap (Fig. 3). Isolation and analysis of the 4 fragments revealed that the various copies of the 7 subunits are symmetrically distributed among the 4 × 6 particle (Markl *et al.*, 1981a). The exact topology was determined by direct localization of subunits in the electron microscope after having decorated the 4 × 6-mer with subunit-specific antibody fragments (Markl *et al.*, 1981d). A ringlike *bc-bc* tetramer forms the central core which connects the hexameric quarter-structures. Presumably, two *f-f* homodimers achieve more peripheral contacts between the 2 × 6-halves. Within each 2 × 6-mer, subunits *a* and *d* are involved in the inter-hexamer connection, whereas subunits *e* and *g* are arranged peripherally. This model of quaternary structure (Fig. 1) is in agreement with a model which was published in the same year for the 4 × 6 hemocyanin of the scorpion *Androctonus australis* (Lamy *et al.*, 1981b).

8 × 6 hemocyanin of the horseshoe crab *Limulus polyphemus* is dissociable into 4 × 6 half-molecules, which morphologically correspond in detail with native *Androctonus* and *Eurypelma* 4 × 6-mers (Bijlholt *et al.*, 1982), and consist of an immunologically related set of subunits (Lamy *et al.*, 1983a; Kempter *et al.*, 1985). Hybrid hemocyanin molecules ("protein chimaeras"), which we obtained by co-reassembly of subunits from the three species, further underlined this structural relationship (van Bruggen *et al.*, 1980). The recently published subunit topology of this largest of all arthropod hemocyanins indeed confirmed its far-reaching homology with tarantula and scorpion hemocyanin (Lamy *et al.*, 1983b).

Hemocyanin subunit structure, and the evolution of spiders

The striking conservatism of the 7 subunit/4 × 6 structure raised the question, what happened with *Cupiennius* to cause such a dramatic change in its design? To find an answer we attempted a broad survey including 40 species of spiders from 25 families (Markl *et al.*, 1983, 1986a). Previous taxonomic schemes for the higher classification of spiders at the family level have been based on adult morphology or behavior, a topic on which there is currently substantial disagreement (Eberhard, 1982). H. W. Levi stated in 1978: "Spider classification at the present is in chaos." (Personal remark, cited in: R. F. Foelix. 1979. *Biologie der Spinnen.* Thieme, Stuttgart). To use spider hemocyanin as a taxonomic character should be advantageous, because in contrast to most other tried characters the direction of its evolution seems clear: the 2 × 6-mer is a derivative of the 4 × 6-mer and not *vice versa,* because the 4 × 6-mer also occurs in other Chelicerata. According to current textbook taxonomy, the order of spiders (Araneae) contains four suborders: Mesothelae (which were not studied by us), Orthognatha (*e.g., Eurypelma*), Cribellata, and Labidognatha. The Labidognatha are further subdivided in Haplogynae (with simple sex organs) and Entelegynae (with complex sex organs). *Cupiennius* is an entelegyne spider.

The four orthognath families studied all possess 4 × 6-mers with a subunit composition corresponding to that of *Eurypelma* hemocyanin; a homologous structure which also occurs in seven entelegyne families (Fig. 4). Eleven other entelegyne families,

however, dispose of hemocyanins which are clearly homologous to that of *Cupiennius* (Fig. 4). Their extreme immunological similarity strongly supports the hypothesis that the last group is a natural, monophyletic taxon. We therefore propose to designate this taxon as "Neo-Entelegynae," and the remaining entelegyne spiders, defined by their 4×6 hemocyanin, as "Arch-Entelegynae" (Markl and Runzler, 1986).

It was very interesting for us to detect in a cribellate spider an intermediate step in the transition from the 7-subunit to the 2-subunit particle: *Filistata insidiatrix* possesses only hexameric hemocyanin (This contradicts Wibo, 1966, who reported 4×6 hemocyanin for this species). The patterns of crossed immunoelectrophoresis showed five subunit peaks (Fig. 4). Absent was heterodimer *bc,* which makes the presence of only hexamers in this spider understandable: in *Eurypelma californicum* 4×6 hemocyanin, *bc* forms a tetrameric core (Fig. 1), which in reassembly experiments was indispensable to exceed the hexamer level (van Bruggen *et al.,* 1980; Markl *et al.,* 1982). *Amaurobius fenestralis,* the second cribellate spider that we investigated, possesses a 2×6 hemocyanin with the typical subunit composition of our Neo-Entelegynae (Fig. 4). Alternatively, the spider *Uroctea durandi,* which morphologically appears to be a very close relative of certain cribellate species (Kullmann and Zimmermann, 1976), belongs to our Arch-Entelegynae according to its hemocyanin structure. The Cribellata possess two unique devices: a typical spinning plate, the cribellum and, at their fourth pair of legs, a special comb to brush silk, the calamistrum. Because a repeated, independent evolution of these structures is highly improbable, most experts treat the Cribellata as a natural, monophyletic taxon (*e.g.,* Bristowe, 1938; Levi, 1966; Lehtinen, 1967). However, the striking morphological similarity between various Cribellata and particular entelegyne species stimulated the idea that cribellate characters have been repeatedly and independently lost in spider evolution (Lehtinen, 1967). Our data strongly support this hypothesis, and indicate that at least all the Neo-Entelegynae, and probably the entire labidognath suborder, stem from cribellate progenitors. Compared to the various schemes based on other characters, our results agree substantially with Lehtinen (1967, 1978), despite minor differences which predominantly are refinements.

The haplogyne spider *Dysdera crocata* possesses a hexameric hemocyanin like *Filistata,* but composed of only two different subunits in comparable proportions; immunologically they correspond to *Eurypelma f* and *d* (Fig. 4). The close relationship to *Cupiennius* hemocyanin is obvious; however, the specific structural role of subunit *d* as hexamer linker (Markl, 1980) is not (yet?) established in *Dysdera.* Thus *Dysdera,* and probably the entire group of haplogyne spiders (although their monophyletic nature is doubted) are either late descendents of a neo-entelegyne spider family, or indeed have conserved molecular features of the transition phase between the two hemocyanins. It should be noted that Lehtinen (1967) postulated a close relationship between Filistatidae and Haplogynae, which supports the second assumption. Although presently we are unable to decide with lasting certainty, whether *Filistata* and *Dysdera* hemocyanin represent true "missing links," they can serve as evolutionary models. It may well be that an ancestral cribellate spider, for whatever reason, lost its ability to genetically express the heterodimer, and thus was restricted to hexameric hemocyanin with possibly drastic (though obviously not lethal!) negative consequences: drop of functional plasticity, increase of colloid osmotic pressure, and blood viscosity. In the descendents of this "genetic cripple," three more subunits (probably unnecessary to bring a hexamer to function) disappeared from the phenotype, and later a new mode of inter-hexamer bridging was invented. This was the starting signal for the evolution of a large variety of highly advanced trappers, hunters, and jumpers (Fig. 4). Whether this new hemocyanin, possibly the result of an evolutionary accident, conceals any functional advantage over the 4×6-mer remains an interesting, unanswered question.

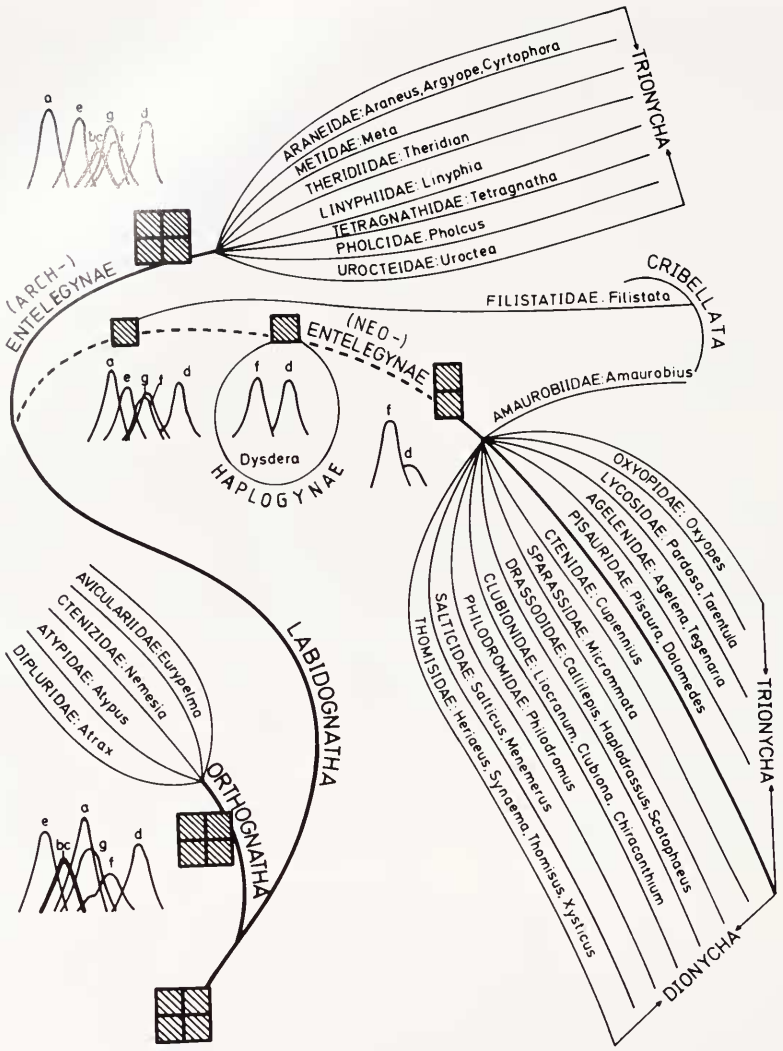

FIGURE 4. Phylogenetic tree of spiders as deduced from their hemocyanin subunit composition. Typical subunit patterns of crossed immunoelectrophoresis, and symbols for the respective native aggregation state (1×6, 2×6, 4×6) are shown. The 7 subunit/4×6-mer occurs throughout the Orthognatha and, immunologically somewhat different though doubtlessly related, in 7 labidognath families, which cover orb-weavers and other stationary trionychan (= three-clawed) species. The 2 subunit/2×6-mer is typical for another 11 labidognath families, including—besides some net spiders (Agelenidae)—a large variety of free hunters: lurking seizers, sudden jumpers, and tenacious runners. Due to the extremely close immunological relatedness of the subunits of all 2×6-mers, these three-clawed (Trionycha) and two-clawed (Dionycha) spiders most probably represent a monophyletic taxon. The transition of 4×6 in 2×6 hemocyanin is conserved, or at least modeled, within the cribellate family *Filistatidae* (*Filistata insidiatrix*) and the haplogyne family Dysderidae (*Dysdera crocata*), which both possess 1×6 hemocyanin, but with a different subunit composition. Note that *Filistata* hemocyanin lacks subunit *bc* which in *Eurypelma californicum* reassembly experiments was indispensable to exceed the 1×6 level (Markl *et al.*, 1982). A second cribellate spider, *Amaurobius fenestralis*, possesses 2×6 hemocyanin.

According to our data, Labidognatha and Cribellata are not separable, but represent phylogenetically intersecting, nested groups. Entelegynae is a polyphyletic taxon, containing two natural groups, which are disconnected by a haplogyne/cribellate cluster. We have therefore proposed to substitute "Entelegynae" for two new monophyletic terms, namely "Arch-Entelegynae" and "Neo-Entelegynae" (Markl and Runzler,

The sea spiders—a phylogenetic mystery?

Besides the aquatic xiphosurans and the terrestrial arachnids, in current taxonomy the subphylum Chelicerata includes as a third class the marine sea spiders (Pycnogonida = Pantopoda), although the phylogenetic position of these indeed spider-like, often incredibly tiny animals is uncertain. Among experts it is still debated whether they are arachnids, non-arachnidan chelicerates, or a completely independent arthropod subphylum (Bergström *et al.,* 1980). Also remarkably, they have no respiratory organs—very unusual among arthropods, especially aquatic species, in which cutaneous gas exchange is blocked by an impermeable exoskeleton. Hoping to detect a phylogenetically interesting new version of the blue protein, we have studied the minute *Nymphon* sp. from the North Sea and the huge Antarctic *Colossendeis* sp. (Markl *et al.,* 1986a; and unpub. data). Unfortunately, polyacrylamide electrophoresis of their blood yielded no trace of hemocyanin-like protein chains.

Earlier, Redmond and Swanson (1968) reported that spectral-absorption curves of the blood of another giant Antarctic sea spider, *Ammothea striata,* showed no evidence of a blood respiratory pigment. *Nymphon* with its size below 1 cm, and body and extremities as thin as threads, is probably qualified for cutaneous gas exchange. However, the absence of an oxygen carrier in *Colossendeis* and *Ammothea,* which both exceed in size a large tropical spider, but live in the oxygen-poor aquatic environment, is surprising. On the other hand, many cold-blooded animals found in polar seas lack respiratory pigments because of the high oxygen solubility in icy-cold body fluids.

Instead of hemocyanin as desired, in both species we observed electrophoretically two prominent polypeptide chains with molecular masses around 110,000—the typical range of the second major blood protein of spiders and other arachnids (left insert in Fig. 2). This protein was described by us in earlier studies and designated as a "non-respiratory protein" (Markl *et al.,* 1976, 1979b; Linzen *et al.,* 1977). In case of the tarantula *Eurypelma californicum,* it was recently further characterized as lipoprotein associated with a carbonic anhydrase (Stratakis and Linzen, 1984). By immuno blotting, indeed a structural relationship between the non-respiratory protein of the tarantula, and the pycnogonid protein was shown (Markl *et al.,* 1986a). According to the available data this protein is restricted to scorpions, whip scorpions, whip spiders, and spiders, and was detected in neither xiphosuran nor crustacean blood; non-respiratory blood proteins of those two groups are composed of considerably smaller polypeptide chains (Markl *et al.,* 1979b, c). Thus, the occurrence of this protein is a good argument in favor of a close phylogenetic relationship between the sea spiders and the arachnids.

Subunit Diversity in Crustacean Hemocyanins

At least 600 million years ago, the branch leading to the Chelicerata was separated from the line leading to the Crustacea (Tiegs and Manton, 1958; Schram, 1982). Since today both groups have hemocyanins based on a hexameric architecture, this hemo-

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1986). Our results agree in principal with the evolution scheme of Lehtinen (1967), which is based on morphological characters.

The figure was slightly modified from Markl *et al.* (1986a). The following species were employed: *Agelena labyrinthica, Amaurobius fenestralis, Araneus diadematus, Araneus umbriaticus, Argiope aurantia, Argiope bruennichi, Atrax formidabilis, Atypus affinis, Callilepis nocturna, Chiracanthium elegans, Clubiona terrestris, Cupiennius salei, Cyrtophora citricola, Dolomedes fimbriatus, Dysdera crocata, Eurypelma californicum, Filistata insidiatrix, Haplodrassus signifer, Heriaeus hirtus, Linyphia marginata, Liocranum rupicola, Menemerus taeniatus, Meta segmentata, Micrommata rosea, Nemesia sp., Oxyopes lineatus, Pardosa amentata, Philodromus collinus, Pholcus phalangioides, Pisaura mirabilis, Salticus scenius, Scotophaeus quadripunctatus, Synaema globosum, Tarentula fabrilis, Tegenaria atrica, Tetragnatha extensa, Theridion varians, Thomisus onustus, Uroctea durandi, Xysticus bifasciatus.*

cyanin undoubtedly belonged to their common ancestor. Thus it was of considerable interest to study the subunit composition of crustacean hemocyanins, which are commonly 1×6 or 2×6 aggregates (the only known exception are the thalassinid shrimps, which possess a tetrahedrical 4×6-mer: Miller *et al.*, 1977; van Bruggen, 1983). We applied high resolution electrophoresis techniques, and revealed complex patterns of subunit heterogeneity in various crustacean hemocyanins: between three and eight distinct polypeptides were detected throughout (Markl *et al.*, 1978, 1979c). It was impossible, however, to recognize a common scheme except for the fact that in 2×6-mers the heterogeneity was somewhat more excessive than in native single hexamers.

To monitor evolutionary trends, we needed a tool to sort all these subunits into groups of similar function. Because protein function is a matter of surface structure, the tool employed was immunochemistry: each antibody is specifically directed against a fragment of the protein surface, and refuses binding if this epitope is altered. The outcome of a comparative immunochemical analysis of 41 crustacean hemocyanins is described below.

Subunit composition of 2×6 hemocyanins from brachyuran crabs

The typical hemocyanin of a brachyuran crab, like the blue crab *Callinectes sapidus,* or the green shore crab *Carcinus maenas,* is a 2×6-mer, composed of two immunologically discernible subunit fractions (Rochu and Fine, 1980; Markl and Kempter, 1981a, b; Ghidalia *et al.*, 1985). We have designated them as alpha and beta (Fig. 5). Beta is immunologically unrelated to alpha, which indicates substantial differences in function. Alpha subunits from different crab species are immunologically similar; they remained relatively unchanged after the worldwide radiation of the Brachyura, and therefore were classified by us as "phylogenetically conservative." In a striking contrast, beta subunits differ greatly immunologically among the species, and therefore were classified as "phylogenetically variable" (Markl and Kempter, 1981a). Obviously, during crab evolution, these two subunit types endured very different selection pressures, which again indicates fundamental differences in function.

All in all, we studied 32 species covering a broad environmental and activity range, from shallow water, intertidal, fresh water, and land, and from most agile to rather clumsy (Markl and Kempter, 1981b; Markl *et al.*, 1986a). The representatives of 10 out of 11 crab families possessed alpha and beta subunits (Fig. 5), regardless of how diverse their subunit patterns were electrophoretically. Many Brachyura also have a third hemocyanin subunit, designated as gamma. Gamma appears to be a late offspring of alpha, because their immunological reactions are similar.

The only exception from this general scheme was detected in the family Ocypodidae: the hemocyanins of a fiddler crab (*Uca urvillei*) and of three species of ghost crabs (Genus *Ocypode*) are exclusively composed of alpha subunits (Fig. 5). It was very interesting to detect that *Uca* hemocyanin particles are only single hexamers, which indicates that the absence of beta may be correlated with a restriction to the hexameric level. However, much to our surprise, all three *Ocypode* species possess mainly 2×6-meric hemocyanin (Stöcker, 1984; Markl *et al.*, 1986a; B. Johnson, pers. comm.). In contrast to *Uca, Ocypode* hemocyanin contains two electrophoretically distinct alpha isozymes (Fig. 5). The cathodic of these immunologically identical proteins migrates in the range of beta, but carries no beta-typical antigen determinants. One could assume that the situation within the Ocypodidae mirrors an ancient trend, leading from single alpha hexamers via alpha/alpha 2×6-mers to alpha/beta 2×6-mers. However since among the Brachyura, *Uca* and *Ocypode* belong to a rather specialized family, other possibilities had to be considered as well. To clarify this point we had to answer the question of whether beta is an invention of the comparatively modern crabs.

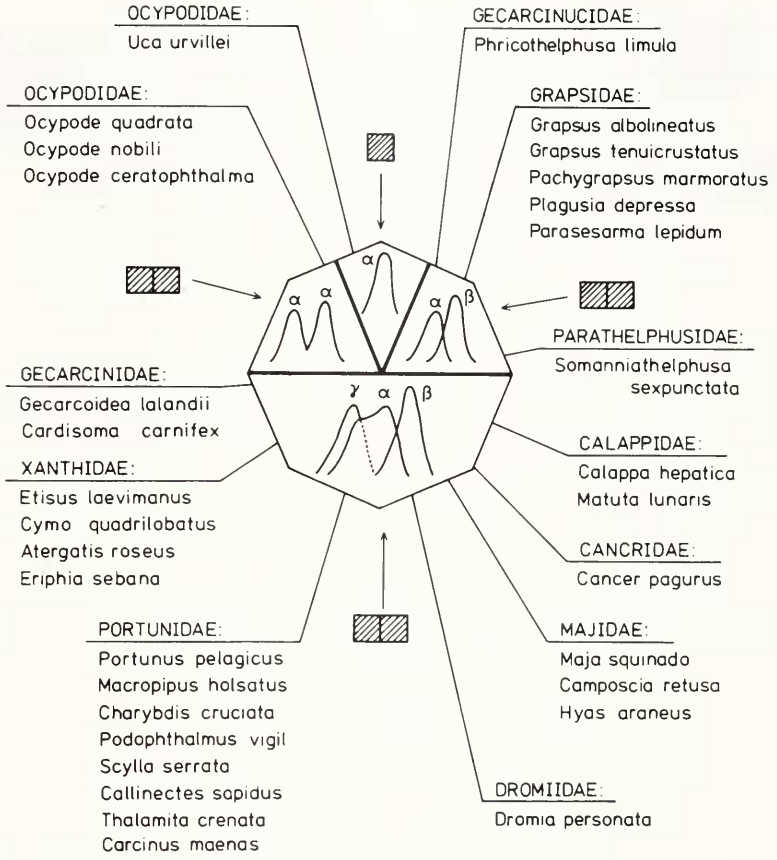

FIGURE 5. Brachyuran crabs arranged according to their hemocyanin subunit composition as analyzed by crossed immunoelectrophoresis. The native aggregation states (1 × 6, 2 × 6) are symbolized. Immunologically corresponding subunits are identically designated. It should be noted that immunologically homogeneous subunit peaks may contain several electrophoretically separated isozymes. Thus, the electrophoretic heterogeneity is generally higher than indicated here (for review: Linzen, 1983).

The tropical species were collected on the Malayan Peninsula. The animals cover a broad environmental range from fairly constant to permanently varying marine *milieus*, from icy to tropic temperatures, and through all stages of land-life: dependent on moist burrows during lowtide (*Etisus*), sun-exposed on rocks (*Plagusia*), in cold freshwater falls (*Phricothelphusa*), in warm stuffy rice fields (*Somanniathelphusa*), in mangrove mud (*Scylla*), in soil burrows (*Cardisoma*), in palmtree forests (*Gecarcoidea*), on hot sandy beaches (*Ocypode*). The smallest (*Cymo*) had a carapace width of less than 2 cm, the largest (*Scylla*) of more than 20 cm. Their activities range from clumsy dwelling (*Dromia*), slow rambling (*Hyas*), hectic rowing (*Matuta*), tenacious waving (*Uca*), fast swimming (*Charybdis*), swift walking (*Carcinus*), and sudden jumping (*Grapsus*), to extremely speedy sand running (*Ocypode*). The figure is taken from Markl *et al.* (1986a).

Spiny lobster 1 × 6 hemocyanin—a possible predecessor?

According to convincing palaeontological records the brachyuran crabs, the latest appearing decapod group, evolved from ancestors related to spiny lobsters (Schram, 1982). Interestingly, spiny lobsters possess 1 × 6 hemocyanin. We investigated the European species *Palinurus vulgaris*, and its American relative *Panulirus interruptus* (Markl *et al.*, 1979c, 1983, 1986a; Stöcker, 1984). Their hemocyanins are composed of an immunologically homogeneous subunit fraction, which corresponds to brachyuran alpha (Fig. 6). *Panulirus* hemocyanin contains a second, cathodic subunit in

addition (designated as *c* by Neuteboom *et al.,* 1986) which, to a certain degree, is immunologically related to alpha. Beta-typical antigen determinants were not detected. This is consistent with our definition of the behavior of a gamma subunit, and the component was designated accordingly, although we have no further evidence that *Panulirus* and brachyuran gamma components are really homologous. From its subunit composition, the hemocyanin of a scyllarid (*Scyllarus arctus*), a close relative of spiny lobsters, fits well into the scheme (Fig. 6); however, surprisingly it forms 2 × 6 particles (unpub.). Although the phylogeny of subunit beta, and also the structural requirements for a 2 × 6 formation remained unclear, the situation found within the Palinura at least further supported the hypothesis that alpha subunits were the basic hexamer formers.

Hemocyanins from lobsters, freshwater crayfishes, and shrimps

Geological strata document that, when the first Brachyura appeared 200 million years ago (their great radiation started 140 million years later upon a worldwide formation of shallow water seas), the Astacura were already established (Schram, 1982). Astacura also possess 2 × 6 hemocyanin. In the case of the European freshwater crayfish *Astacus leptodactylus* it is composed of four distinct subunits: a disulfide bridged dimer and three monomers (Markl *et al.,* 1979c; Pilz *et al.,* 1980; Markl and Kempter, 1981b).

Without major difficulties, the dimer and two of the three monomers could be assigned to the alpha/gamma cluster of the Brachyura (Fig. 6). Again, the conservative nature of alpha-typical antigen determinants was confirmed (Stöcker, 1984; Markl *et al.,* 1986a). What remained was the question of a possible relationship between the third *Astacus* monomer and brachyuran beta. By crossed immunoelectrophoresis, anti-*Astacus* antiserum did not precipitate *Cancer pagurus* beta, and *vice versa.* Therefore, for a comparison of structurally distant antigens we applied the immuno blotting technique, which is more efficient. This sensitive method revealed that anti-*Astacus* antibodies specific for the third monomeric subunit preferentially bind to brachyuran beta and *vice versa,* whereas alpha components are only weakly recognized (Markl *et al.,* 1986a). Comparable data stem from the 2 × 6 hemocyanin of the lobster *Homarus americanus,* although in this case the alpha fraction was immunologically homogeneous and no dimer was present; therefore we could not define a subunit alpha′ in this animal (Fig. 6). Hemocyanin from another decapod, the caridean shrimp *Palaemon elegans,* contains only alpha and gamma subunits (Stöcker, 1984; Markl *et al.,* 1986a); however, its native aggregation level is 1 × 6 (Fig. 6)! These results indicated: first, beta is not an invention of the Brachyura, but phylogenetically considerably older, and second, the presence of beta is correlated with the appearance of 2 × 6 particles. The second conclusion is implicated by the exceptions *Ocypode* and *Scyllarus,* which will be discussed below.

It should be noted that in shrimps and crayfishes, gamma subunits are only defined according to their close relationship with alpha subunits; in contrast to alpha and beta, the homology of astacuran or caridean gamma with the respective palinuran and brachyuran components is uncertain. Gamma could have evolved independently from alpha several times.

The origin of subunit beta

It was found recently that 2 × 6 hemocyanin also occurs in a fourth decapodan group, namely the Anomura (Stöcker, 1984; Markl *et al.,* 1986a). Surprisingly, despite a marked electrophoretic heterogeneity, the subunits of 2 × 6 hemocyanins from a galatheid shrimp (*Galathea squamifera*), a hermit crab (*Pagurus bernhardus*), and the coconut crab (*Birgus latro*) were immunologically completely homogeneous (Fig. 6).

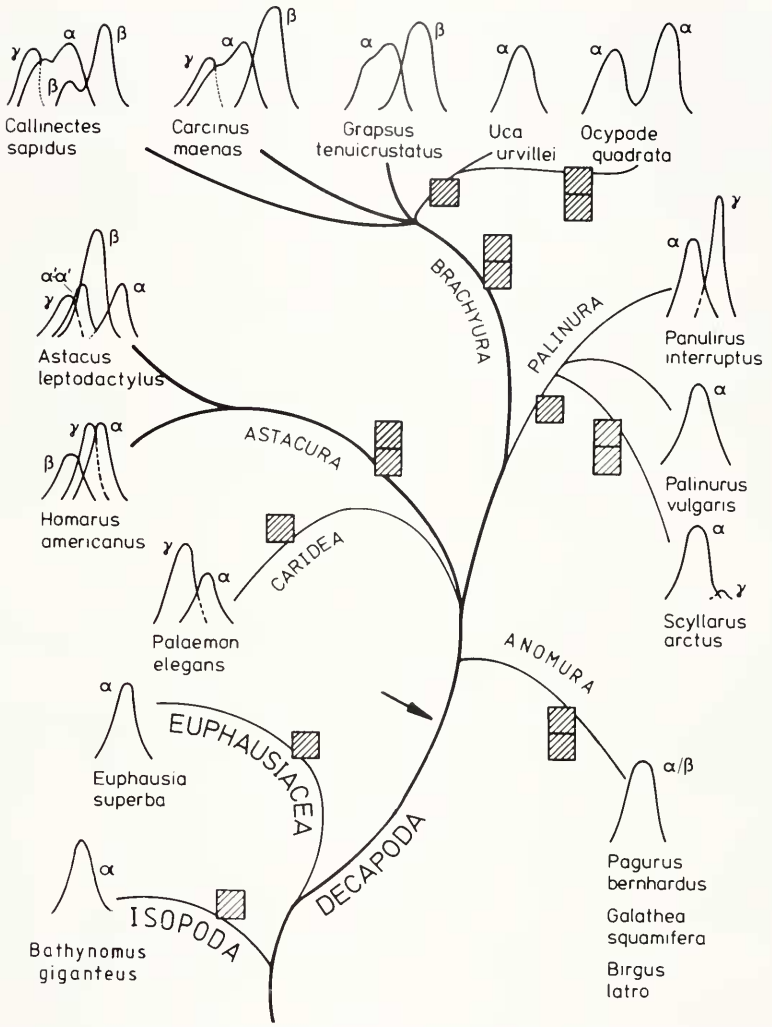

FIGURE 6. The distribution of immunologically corresponding hemocyanin subunits among the Crustacea. The various subunit patterns of crossed immunoelectrophoresis are shown, and the native aggregation states (1×6, 2×6) are indicated.

It appears that decapodan hemocyanins evolved from an ancient alpha hexamer; the first step was the invention of beta-typical antigen determinants (arrow), which is correlated with the occurrence of 2×6 aggregates. Beta was repeatedly lost in later appearing groups (Caridea, Palinura, *Uca/Ocypode*). All alpha subunits are homologous proteins as are all beta subunits. Gamma differs, however: except for their partial identity with alpha, we were unable to identify typical immune reactions which interpose between more distant gamma subunits. Thus, rather than being homologous, they could represent convergent offsprings of different alpha progenitors.

It should be noted that our results, although useful in explaining the phylogenetic coherence between the various subunits, in most cases are insufficient to refine the current phylogenetic tree derived from morphological characters and palaeontological records (Schram, 1982), which is the basis for this scheme. As an exception, our data strongly support an early branch of the Anomura as indicated, which was not derived from classical approaches. The figure was modified from Markl *et al.* (1986a).

Thus, in terms of immunochemistry, only one single subunit type is present. We obtained clear immunological cross-reactions with brachyuran and astacuran alpha but also, and this was the second surprise, with astacuran (not with brachyuran!) beta. The common presence of alpha- and beta-typical antigen determinants on a single subunit cannot be explained as the result of a gene fusion, because the size of the anomuran polypeptide chains is quite within the expected range (Mr = 76,000–83,000). Provided that hemocyanin genes are arranged in a single cluster, one could expect a current rearrangement by unequal crossing-over, which may well explain the anomuran result. However, the marked divergence between alpha and beta in crabs and crayfishes strongly indicates the existence of two different gene clusters like, for example, the case with the mammalian globin genes. It is therefore much more likely to assume that in the Anomura the blue protein has preserved an ancient feature, and that in later appearing species, alpha and beta antigen determinants have been separated by the independent evolution of isozymes. This strongly suggests that the evolution of the decapod 2 × 6-mer indeed began with the appearance of subunit beta, although later beta was repeatedly lost (Palinura, Caridea, Ocypodidae). This, however, was not necessarily tantamount to a permanent restriction to the hexameric level, because alternative, beta-free modes of 2 × 6-mer formation evolved in specialized groups (*Ocypode, Scyllarus*).

Krill and isopod 1 × 6 hemocyanin: models of an ancient design

We tried to demonstrate this by examining the situation outside of the Decapoda. The widespread hemocyanin aggregate of the other Malacostraca is the 1 × 6-mer, which we studied from the Antarctic krill *Euphausia superba* and from the giant deep sea isopod *Bathynomus giganteus* (Fig. 6). The subunits of both proteins exhibited a completely homogeneous peak in crossed immunoelectrophoresis, and corresponded immunologically to subunit alpha of decapodan hemocyanins. Thus, those 1 × 6-mers are entirely composed of alpha subunits (Fig. 1; the different information of Markl *et al.*, 1983, was due to denaturation). It appears, moreover, that *Euphausia* hemocyanin has maintained a considerably ancient surface structure: by immunoelectrophoresis, it was the only crustacean hemocyanin which could be precipitated (in its oligomeric form—not as subunits: see below) by an antiserum raised against tarantula hemocyanin (Van Holde and Brenowitz, 1981; M. Brenowitz, pers. comm.; Stöcker, 1984; Markl *et al.*, 1986a, and unpub. data). This definitely showed that, in arthropods, alpha-typical antigen determinants form the ancestral design of the blue protein's surface.

Mirrors larval crab hemocyanin the blue protein's evolution?

The subunit composition of crab hemocyanins have endured ontogenetic changes (Terwilliger and Terwilliger, 1982). We therefore studied various larval stages of the spider crab *Hyas araneus* and the shore crab *Carcinus maenas.* Hemocyanin from the planktonic zoea, and from the benthonic megalops of both species was composed of a single subunit type, which clearly cross-reacted with adult alpha subunits (Markl *et al.*, 1986a), although electrophoretically it behaved somewhat differently. The native aggregation level of those larval hemocyanins is mainly hexameric, but also 2 × 6-mers have been observed in the electron microscope (unpub.). Thus, the loss of subunit beta in the evolution of some decapoda, and an alternative mode of 2 × 6-mer formation (*Ocypode, Scyllarus*), could have occurred to preserve larval characters.

Recently, we have further monitored the following events in *Carcinus maenas:* after metamorphosis, during the following molting cycles subunit beta appears, although its proportion in the first juvenile crab is still rather low. 2 × 6 hemocyanin particles can be seen in the electron microscope, but the main aggregation level is still

hexameric. The second juvenile crab clearly contains mostly 2 × 6 hemocyanin with a considerable proportion of subunit beta. Subunit gamma appears several stages later when the carapace measures 12 mm in width. The typical, stoichiometrically correct subunit pattern of the adults is established when the carapace width measures 22 mm or more (L. E. Precht, B. Steiff, and J. Markl, unpub. data). It is interesting that not only phylogenetically, but also ontogenically, alpha subunits appear first. Also our above presumption that gamma may be a rather late evolutionary product is mirrored in ontogenesis.

A model of crustacean 2 × 6 hemocyanins

The results described above indicate that particular subunits play distinct roles in the formation of 2 × 6 structures. This was closely investigated in two astacuran and two brachyuran species. The overall morphology of these particles, especially the one-point contact between the two hexamers as shown in Figure 1, is described by van Bruggen (1983). Reassembly experiments with isolated subunits alpha, beta, or gamma of the 2 × 6 hemocyanins from the crayfishes *Astacus leptodactylus* and *Homarus americanus*, and from the crabs *Cancer pagurus* and *Callinectes sapidus* showed that in most of the cases, homo-hexamers can be formed (Markl and Kempter, 1981a; Stöcker *et al.*, 1986). However, all three immunologically defined classes of subunits are required to reach the 2 × 6 level (Stöcker *et al.*, 1986). Immunologically identical isozymes are in some cases capable of substituting for each other, especially in *Homarus* and *Callinectes*. Heptameric segments of native 2 × 6-mers were observed in *Astacus*, whereas the 2 × 6 particles of crabs and *Homarus* dissociate via hexamers (Markl *et al.*, 1981c). The inter-hexamer bridge was identified in the case of *Astacus* and *Cancer*. Surprisingly the bridge is not formed by subunit beta as expected. In *Cancer* a particular alpha isozyme (designated as alpha′) has a tendency to dimerize, and connects the two hexamers (Markl *et al.*, 1983). Correspondingly, in *Astacus* the disulfide-bridged dimer is a correlate of alpha. This makes the existence of beta-free 2 × 6 structures as present in *Ocypode, Scyllarus*, and in larval crabs, at least structurally understandable. It should also be noted that 2 × 6 hemocyanins were reported for two species outside of the Decapoda: the isopod *Ligia pallasii*, and the stomatopod *Squilla mantis* (Terwilliger, 1982; van Bruggen, 1983). From our data described above, we would expect a beta-free mode of 2 × 6 formation also in those hemocyanins.

Our next step was to analyze the respective subunit stoichiometries. Finally, labeling of the dodecamers with subunit-specific antibody fragments, or with intact antibody molecules, and observation of the resulting complexes in the electron microscope led to a uniform topological model (Fig. 1). It shows that one dimer alpha′-alpha′ and four copies of beta form a central cluster. Although beta may not be involved directly in the bridge, these four subunits are clearly in the topographical position to play a functional key-role. One alpha occupies the extreme outer edge of each hexamer, and four copies of gamma fill the periphery (Stöcker *et al.*, 1986). Though a preliminary topological model of a crustacean 2 × 6 hemocyanin was already published by Jeffrey (1979), this is the first detailed conception of its architecture.

The connection to the cheliceratan subphylum

We were particularly interested in detecting immunological relationships between crustacean hemocyanin subunits and those from the Chelicerata. By immunoelectrophoresis, a precipitation of crustacean hemocyanins with an anti-chelicerate antiserum, or *vice versa*, was only successful if the hemocyanin was either present in its oligomeric form (see above: *Euphausia*), or denatured in 8 *M* urea (Stöcker, 1984). Therefore, we performed immuno blotting experiments with electrophoretic patterns of native hemocyanin subunits from the horseshoe crab *Limulus*, the scorpion *Androctonus*, the tarantula *Eurypelma*, and the hunting spider *Cupiennius* against anti-alpha antisera

from crustaceans. In each case, the total set of chelicerate subunits was recognized (Markl *et al.,* 1986a). Anti-beta and anti-gamma antisera give similar, but much weaker reactions. As judged semiquantitatively, chelicerate subunits related to *Eurypelma a, d,* and *f* are recognized best. This is illustrated in the right insert of Figure 2. It should be interesting to analyze comparatively the 6 × 6 hemocyanin of the myriapod *Scutigera coleoptrata* described by Mangum *et al.* (1985). Our results do *not* mean that there is one particular chelicerate subunit which corresponds to alpha, and another subunit which corresponds to beta; those specializations are certainly late developments. We showed that despite their impressive diversity in recent species, the hemocyanin subunits of all arthropods have maintained—to a different extent—some common ancient surface structures. These features were inherited, unchanged, over at least 600 million years!

Interaction of Subunits in the Oxygen Binding Process

Recently, several complete amino acid sequences of chelicerate and crustacean hemocyanin subunits were published, the structure of the active copper-site was further elucidated, the conformation of a crustacean 1 × 6-mer was studied in a 3.2 Angström resolution, and the topologic models of *Limulus* and *Androctonus* hemocyanin were considerably refined (Solomon, 1981; Sizaret *et al.,* 1982; Schartau *et al.,* 1983; Schneider *et al.,* 1983; Eyerle and Schartau, 1985; Gaykema *et al.,* 1985; Lamy *et al.,* 1985; Linzen *et al.,* 1985). This information, in the context of the results described here, has demystified, to a considerable degree, the architecture and the evolution of arthropod hemocyanins. Moreover, a wealth of data exists on their function as oxygen carriers (*e.g.,* Van Holde and van Bruggen, 1971; Bonaventura and Bonaventura, 1980; Mangum, 1980, 1983, 1985; Van Holde and Miller, 1982; Antonini *et al.,* 1983; Bridges *et al.,* 1983; Ellerton *et al.,* 1983). However, the processes which determine the typical oxygen binding, and the specific contribution of each subunit type, are still obscure. Presently, *Eurypelma californicum* hemocyanin is the best understood example.

The 4 × 6-mer in action, and the abilities of isolated subunits

Hemocyanin from the tarantula *Eurypelma californicum* is characterized by a relatively low oxygen affinity, a strong normal Bohr effect (= pH sensitivity of oxygen affinity), and an extreme cooperativity (sigmoidity of the oxygen binding curve); additionally, the cooperativity depends considerably on pH (Fig. 7). Upon direct measurements of blood pH and blood P_{O_2} in different tissues during exercise and rest, these properties can be interpreted as highly adaptive with respect to the animal's environment, behavior, and physiology. (Linzen *et al.,* 1977; Loewe, 1978; Angersbach, 1978; Decker *et al.,* 1983a, b; Fincke *et al.,* 1986; Paul, 1986). In contrast, as illustrated in Figure 7, isolated subunits are non-cooperative (hyperbolic binding curve), show a high oxygen affinity, and indicate a complete absence of any Bohr effect. All seven subunits, studied individually, behaved rather uniformly in those aspects (Decker *et al.,* 1979; Markl *et al.,* 1981b, e). Thus, the 4 × 6-mer acts as a system; all its vital abilities are qualitatively new and unexpected, and cannot be predicted from the properties of the subunits. The systemic characters are created by an interaction of the 24 constituents. With subunits in the blood instead of 4 × 6 particles, the animals could impossibly survive. An interesting question was now, at what structural level the respective function occurs?

Events within and beyond the native 1 × 6 fragment

Tarantula hemocyanin again was an exceptionally useful molecule, because the four oligomeric dissociation fragments described above (Fig. 3) could be stabilized in their respective structure over a broad pH range—a fundamental requirement for a

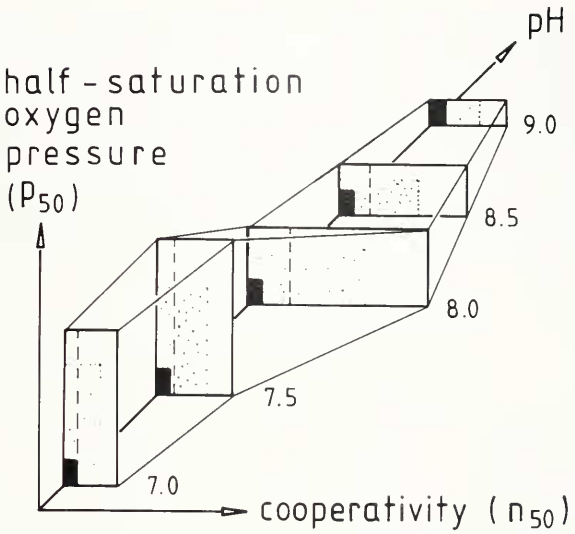

FIGURE 7. Illustration of the functional plasticity of native 4 × 6-meric hemocyanin of the tarantula *Eurypelma californicum*, compared to the functional inflexibility, and the very different behavior, of the isolated subunits.

Subunits (*small black areas*) show a low p50 around 5 mm Hg, a high oxygen affinity. This behavior is independent of pH (= no Bohr effect). Moreover, they are non-cooperative (hyperbolic oxygen binding curve: n50 = 1). All subunits behave similarly (Decker *et al.*, 1979; Markl *et al.*, 1981e). In contrast, the 4 × 6-mer (*large white rectangles*) shows p50 values up to 30 mm Hg (at pH 7.5), or low oxygen affinities. Moreover, it exhibits impressive cooperativities (sigmoid oxygen binding curves); the maximum at pH 8.0 goes up to n50 = 8 and more. Oxygen affinity, and also cooperativity, are *both* strongly pH dependent (Loewe, 1978), which is highly adaptive with respect to the animal's physiology and behavior (Angersbach, 1978). We recently detected that, although the natural resting blood pH of the tarantula is 7.5 (Angersbach, 1978), the *in vivo* behavior of the 4 × 6-mer should be like that illustrated here for pH 8.0; calcium and magnesium ions in native concentrations (4 m*M* each) modulate the function correspondingly (B. Markl and J. Markl, unpub.). All in all, this aggregate of 24 functionally limited components displays completely novel physiological properties. These systemic characters are created by subunit interaction phenomena, and cannot be predicted from the behavior of the isolated subunits.

To study the molecular organization of those interactions, one attempt is to analyze the abilities of oligomeric dissociation fragments (Savel *et al.*, 1983, 1986): for example in the 1 × 6 quarter-structure (areas on the left of the *dashed lines*), the 4 × 6-mer's oxygen affinity is already fully established, but cooperativity reaches only ¼ of the final values.

Another attempt is to analyze reassembled 4 × 6-mers which have a chemically modified subunit incorporated. Modification was done, for example, with mercury(II) ions. A treatment of the whole molecule blocks the interaction processes entirely: the morphologically intact 4 × 6-mer functions like isolated subunits (Markl *et al.*, 1986b). Hemocyanins composed of one mercury-labeled and six unmodified subunit types show a reduced functional plasticity (*spotted areas*), which is believed to be due to the encoupling of the modified subunit from the interaction processes (Markl *et al.*, 1986b).

detailed comparison of their function. Another chelicerate, *Limulus polyphemus*, unfortunately could not fulfill this demand (Brenowitz *et al.*, 1984). In contrast, 1 × 6 hemocyanin fragments of certain crustaceans, like the lobster *Homarus americanus*, the thalassinid shrimp *Callianassa californiensis*, and in the mangrove crab *Scylla serrata*, are stable (Tai and Kegeles, 1971; Arisaka and Van Holde, 1979; Herskovits *et al.*, 1983; Decker *et al.*, 1986a).

An oxygen binding analysis of the isolated quarter-structure (1 × 6-mer) of *Eurypelma* hemocyanin revealed that already on this level, the typical oxygen affinity and the full Bohr effect of the 4 × 6-mer are established; however, cooperativity reaches only 25% of the native value (Fig. 7; Savel *et al.*, 1983). The heptamer equals the hexamer in these properties, but in the half-molecule (2 × 6-mer) cooperativity abruptly

rose to 50% of the end value. The 19-meric fragment provides no further enhancement; for the jump to 100% the entire 4 × 6-mer was required (Savel *et al.*, 1986). These results are consistent with a recently introduced theoretical description of the oxygen binding process, the so-called "nesting model," an extension of the classical "MWC" model (Decker *et al.*, 1986b). This new model is based on the idea of Wyman (1984) that cooperative oxygen binding manifests itself by a hierarchy of nested allosteric units—a theoretical approach which was experimentally proved here not only for the first hemocyanin, but generally for the first allosteric macromolecule. Moreover it was interesting that the ultimate creator of all studied systemic characters is indeed the hexamer; the higher structural levels only quantitatively improve effects.

Installed subunits act as amplifiers and transmitters

We have started to characterize specific roles of the various subunits in the overall oxygen binding process, because their uniformity in the isolated state does not necessarily mean that they behave identically when incorporated in the 4 × 6 particle. Our current project deals with the analysis of reassembled 4 × 6-mers which have one chemically modified subunit type. First, we had to establish the gentle immuno affinity chromatography for subunit purification to prevent the loss of important aspects of function due to uncontrolled protein damage later in the reassembled molecules.

We have already analyzed reassembly products with incorporated "apo-subunits" (copper-free), or "met-subunits" (copper oxydized), but most of our data stem from experiments with "mercury-subunits": Mercury(II) ions undialyzably bind to tarantula hemocyanin in an amount of 1–2 atoms per subunit. The effect of a mercury treatment of the whole 4 × 6-mer is dramatic: it is still a 4 × 6-mer, and still binds oxygen, but the binding properties correspond to those of single subunits (Markl *et al.*, 1986b). This means that all subunit interactions are totally blocked. Comparable behavior is exhibited by *Limulus polyphemus* and *Callinectes sapidus* hemocyanin (Brouwer *et al.*, 1983). Correspondingly, the treatment of a subunit with mercury, followed by its reincorporation into the 4 × 6-mer, should result in an uncoupling of the interaction processes. In such experiments indeed in the case of all subunits, alterations of oxygen affinity, cooperativity, and Bohr effect were monitored (Fig. 7). According to the data, a relatively uniform role as amplifiers can be ascribed to the various monomeric subunits. Heterodimer *bc* also amplifies, and additionally seems to function as a molecular transmitter between the four hexamers (Markl *et al.*, 1986b). This is the first information about the specific contribution of individual subunit types to the allostery of any one hemocyanin molecule.

Future Aspects

Despite our new understanding of the structure, function, and evolution of subunit diversity in arthropod hemocyanins, there remain many challenging questions. For example, tarantula hemocyanin displays its cooperativity maximum at pH 8.0, but the resting blood pH of the animal as measured *in vivo* is 7.5, and drops to 7.0 after activity (Angersbach, 1978; Loewe, 1978). In this range, however, cooperativity does not convincingly exceed the level already achieved (at least at pH 8.0) by the isolated 1 × 6 quarter-structure (Fig. 7). For what reason does a complicated, *bc*-controlled interaction between the four hexamers actually exist, if it is not, or not fully, utilized by the animal? Very recently, we discovered that the whole problem was due to our incomplete knowledge of the actual situation: calcium and magnesium ions in their native blood concentrations (4 m*M* each: Schartau and Leidescher, 1983) enhance, at pH 7.5, cooperativity to the maximum level (B. Markl and J. Markl, unpub.). It should therefore be interesting to investigate the influence of these ions, and of other modulators, on particular subunits.

Other important problems related to subunit diversity are the biological significance of changes in subunit composition while crustaceans adapt to different environments

(Mason *et al.,* 1983), the significance of ontogenetic changes, the significance of the interspecific variability of subunit beta, and similar projects. Another open field is the observation of conformational changes in certain subunits during oxygen binding, which presently is being attempted in our laboratory using fluorecent probes (Leidescher and Linzen, 1986). Although hemocyanin c-DNA already could be cloned, and sequenced (Voit and Schneider, 1986), the cytoplasmatic events in the hemocyanin synthesizing cells are still unknown. Those "cyanocytes" (Fahrenbach, 1970) proliferate in crabs from lymphocytogenic nodules in the outer gizzard wall and in spiders from the inner heart wall (Ghiretti *et al.,* 1977; Kempter, 1983). One of our most important goals is to delve into the cyanocytes, to analyze the location, structure, and organization of hemocyanin genes, which are still entirely concealed, and to reveal how the expression of the diverse subunits is regulated.

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